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Temperature-induced sex reversal is not responsible for sex ratio distortions in grayling *Thymallus thymallus* or brown trout *Salmo trutta*

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Abstract

Based upon experiments carried out over various years, it was concluded that (i) grayling *Thymallus thymallus* and brown trout *Salmo trutta* are resistant to temperature-induced sex reversal at ecologically relevant temperatures; (ii) environmental sex reversal is unlikely to cause the persistent sex ratio distortion observed in at least one of the study populations, and (iii) sex-specific tolerance of temperature-related stress may be the cause of distorted sex ratios in populations of *T. thymallus* or *S. trutta*.

Key words: climate change; salmonid; environmental sex reversal

Water temperature affects behaviour, physiology, and development of aquatic ectotherms (Haugen & Vøllestad, 2000) and could therefore influence key demographic aspects like population sex ratio. Water temperature could, for example, influence sex-specific mortality or even induce sex reversal. The latter is possible in many fishes and amphibians where sex is determined by an interaction between genetic and environmental factors, and temperature often plays a critical role (Devlin and Nagahama, 2002; Baroiller *et al.*, 2009; Stelkens & Wedekind, 2010).

In salmonids, i.e. the family Salmonidae within the order Salmoniformes, sex seems primarily genetically determined (Davidson *et al.*, 2009, Yano *et al.*, 2013). However, several studies found evidence for temperature-induced sex reversal within the genus *Oncorhynchus* (Craig *et al.*, 1996; Azuma *et al.*, 2004; Magerhans *et al.*, 2009). Distorted population sex ratios have been reported in other genera, including *Salmo* (Consuegra & de Leaniz, 2007) and *Thymallus* (Wedekind *et al.*, 2013). In the latter case, the operational sex ratios in a pre-Alpine population varied around 65% males from 1948 until 1992 and shifted to around 85% males from 1993-2011, that is starting five

years after the abrupt shift in water temperatures across rivers and streams in Central Europe (Hari *et al.* 2006). Indeed, there was a statistical link between the water temperatures the fish experienced during their first year of life and the operational sex ratio of adults at an average age of five years. Nevertheless, as Ospina-Álvarez & Piferrer (2008) pointed out, temperature effects on sex determination could be less widespread than previously believed, and experiments within ecologically relevant temperatures are necessary to establish possible links between, for example, climate change and temperature-induced sex reversal. Moreover, Magerhans *et al.* (2009) found both maternal and paternal effects on family sex ratios in rainbow trout *Oncorhynchus mykiss (*Walbaum 1792). Such effects could be linked to phenotypic variation in, for example, the timing of spawning, which has been shown to create sex-specific selection differentials in other taxa (Conover, 1984). Therefore, tests on the effect of temperature on population sex ratio should (i) be based on samples that represent the genetic diversity within natural populations, (ii) compare different phenotypic clusters within populations, and (iii) test for possible differences in family sex ratios.

Wild genitors were collected during spawning seasons of 2007 until 2011. Grayling *Thymallus thymallus* L. 1758 were sampled in March either from the River Aare at the outlet of Lake Thun (the population described in Wedekind *et al.* 2013), or from the River Rhine at the outlet of Lake Constance, Switzerland. Brown trout *Salmo trutta* L. 1758 were sampled in November from five different locations, including the River Aare and four of its tributaries as described in Pompini *et al.* (2013). Gametes stripped from the adults were used for full-factorial *in vitro* fertilizations (methods described in von Siebenthal *et al.*, 2009) each in order to maximize the genetic diversity within the F1 (details in Supplementary Table I). Embryos were either raised at typical hatchery conditions in spring water in trays of vertical flow incubators (study in 2007), or singly until hatching in 2 ml wells (24-well plates; Becton-Dickinson; www.bdbiosciences.com) in chemically standardized water as in Jacob *et al.* (2010) in all studies from 2008 on. After hatching, larvae were raised either in 1500-litre outdoor tanks (spring water; study in 2007), or in 200-litre aquaria within climate rooms (tab water, 12 h daylight cycles) and until dissection (studies in 2008 and 2009), or in 200-litre aquaria first and, after they had reached on average about 2 cm length, in 1500-litre outdoor tanks (sand-filtered lake water; studies in 2010 and 2011). Larvae were first fed with live zooplankton (mostly copepods) and, after they had reached about 2 cm in length, increasingly with dry food (Skretting, Nutra Brut 3.0, 2.0, T-1.1; www.skrettingnwe.com).

In general, the F1 from each breeding experiment were equally distributed to a warm and a cold environment and raised until sex could be determined in a subsample via macroscopic examination of the gonads and histological preparations in case of uncertainty (Guerrero & Shelton, 1974; Supplementary Figure 1), i.e. for at least half a year. In order to reconstruct the natural temperature environment that *T. thymallus* of the River Aare were exposed to from 1971 until 2011, continuous recordings of water temperature at the spawning site and the information about the spawning season given in Wedekind & Küng (2010) and Wedekind *et al.* (2013) were used. The exact spawning time of *T. thymallus* from the River Rhine is not known, but the available recordings of water temperature at their spawning site and the days ripe spawners could be sampled during the previous years suggest that their natural temperature environment was comparable to the River Aare. In the case of *S. trutta*, the initial drop and later raise of temperature that embryos would naturally be exposed to during winter (Renata Hari & C. Wedekind, unpublished results) was simulated in two scenarios within the range of

expected seasonal temperatures. Supplementary Figure 2 shows the range of water temperatures that naturally spawned *T. thymallus* embryos and larvae are exposed to in the river Aare, and Supplementary Table I summarizes the temperature conditions in the various experiments. Of 16 different treatment groups in total, two were lost due to accidents not related to the experimental conditions, while cold incubation temperatures seemed to be linked to increased mortality in others, leading to comparatively small sample size of n=27 and 34 in two groups, while n was always ≥ 100 in the remaining 12 groups (Supplementary Table I).

In 2010, potential paternal and maternal effects on family sex ratio were tested in a common garden experiment, i.e. all F1 were pooled and distributed among either a warm or a cold treatment (Supplementary Table I). After sexing, DNA was extracted from fin clips using the QIAamp DNA Mini Kit (Qiagen Inc.; www.qiagen.com) following manufactures instructions. Fifteen microsatellite markers were used to determine parental identity: *BFRO004, BFRO005, BFRO010, BFRO011, BFRO013, BFRO015, BFRO017, BFRO018* (Koskinen & Primmer, 2001) and *BFRO006, Ogo2*, *SSOSL311, F43, Ocl8, One2*, *One8* (Gum *et al.*, 2003). Multiplex PCR amplification was optimized to be performed in a 10µl reaction volume containing 5-10 ng of DNA, 5µl HotstarTaq master mix (Qiagen, Cat. No 203445), double distilled water, and 0.3-0.6µM of forward and reverse primers each. The following thermo treatment on a TC-412 Programmable Thermal Controller (Techne; www.techne.com) was used: 35 cycles with 94°C for 30 seconds, 56°C for 90 seconds, and 72°C for 60 seconds. Before the first cycle, a prolonged denaturation step (95°C for 15 min) was included and the last cycle was followed by a 30 min extension at 72°C. PCR products were analyzed with an ABI PRISM 3730 genetic analyzer (Applied Biosystems; www.appliedbiosystems.com) using the GeneMarker® Software v1.80 (SoftGenetics LLC®; www.softgenetics.com). Parental identity was established using the CERVUS program 3.0.3 (Marshall *et al.*, 1998).

In order to test for a potential effect of early versus late spawning on offspring sex ratio, *T. thymallus* genitors were collected on two different days in 2011, the first at the beginning of the breeding season and the second at the end of the season, simulating the temperature regime the offspring would usually be exposed to (Supplementary Table I).

Statistical analyses were performed with the open-access software R (R Development Core Team, 2011; www.r-project.org). A generalized linear model was used to analyse maternal, paternal, and temperature treatment effects (all fixed) on offspring sex. The sire x dam interaction term was omitted because of low sample size per experimental cell. All P -values are two-tailed.

Sex ratios did not differ from 50:50 in any of the 12 different thermal conditions that the *T. thymallus* embryos and larvae had been exposed to (binomial tests, P always ≥ 0.25). The 12 tests include the outcome of early and late spawning, i.e. the timing of spawning did not significantly affect offspring sex ratio. Likewise, sex ratios of *S. trutta* raised under two different experimental conditions (Supplementary Table I) were not significantly different from a 50:50 distribution.

In total 495 *T. thymallus* juveniles from two thermal conditions were successfully genotyped. The loci BFRO015, Ocl8, and One8 were monomorphic, the other 12 microsatellites were used to assign juveniles to dams and sires with an average confidence of $P < 0.05$ in 95.6% of the individuals. The number of offspring assigned to the 6 dams ranged from 53 to 147, and the number of offspring assigned to the 20 sires ranged from 12 to 35. No parental effects on family sex ratio were found (Table I).

A close to 50:50 sex ratio can be expected at conception in species with genetic sex determination. Distorted population sex ratios are then either due to sex-specific mortality or environmental sex reversal. The latter has been observed repeatedly within the genus *Oncorhynchus:* environmental sex reversal within this taxon can be induced by temperature (Craig *et al.*, 1996; Azuma *et al.*, 2004; Magerhans *et al.*, 2009) or by hormone-active substances (van den Hurk & Slof, 1981; Hunter *et al.*, 1986). Because sex chromosomes in fish are usually little degenerated (Schartl, 2004), the mismatch between genotype and phenotype created by environmental sex reversal can lead, for example, to XY-XY crossings and the creation of YY individuals who would only produce offspring with a male genotype. This can amplify possible sex ratio distortions or can lead to other kinds of population sex ratios over time (Cotton & Wedekind, 2009). It can even lead to the extinction of sex chromosomes (Cotton & Wedekind, 2009). Distorted sex ratios have been repeatedly reported in salmonid populations (Consuegra & de Leaniz, 2007) and could be contributing to population declines (Wedekind, 2012).

Not much seems to be known about environmental sex reversal in the other taxa within the salmonids (Davidson *et al.*, 2009). Wedekind *et al.* (2013) found persistently unequal sex ratios in one of the study populations. They argued that chemical pollution is unlikely to explain their observation, while population sex ratios could be statistically linked to the temperatures the animals experienced during their first spring and summer. Wedekind *et al.* (2013) concluded that the distorted sex ratio is most likely due to sex-specific tolerance of temperature-related stress or due to temperature-induced sex reversal. The latter was tested for here but no evidence for temperature-induced sex reversal was found in *T. thymallus*.

The subfamily Thymallinae forms the more ancestral group within Salmonidae, with Coregoninae and Salmoninae as sister groups (Koop *et al.*, 2008). *Salmo trutta* is taxonomically closer to the genus *Oncorhynchus* as it belongs to the same subfamily (Salmoninae). However, the common ancestor of *Salmo* and *Oncorhynchus* is estimated at some 15-20 million years ago (Esteve & McLennan, 2007). No evidence for temperature-induced sex reversal was found in *S. trutta*, either. This suggests that *Oncorhynchus* has evolved towards a more labile sex determination system than other salmonids.

No significant deviation from a 50:50 sex ratio was found in any of the 14 ecologically relevant temperature experiments that the fish were successfully exposed to over five consecutive years. This suggests that there are no population differences in juvenile sex ratios under laboratory conditions, and that the conditions the fish were raised in did not cause sex-specific mortality. Hence, if sex-specific mortality explains distorted sex ratios in the wild, it is most likely linked to factors that were excluded in the laboratory such as, for example, pollution (Afonso *et al.*, 2003), certain kinds of parasites or pathogens that could create sex-specific selection (Pickering & Willoughby, 1982; Poulin & Thomas, 1999), or sex-specific predation. The findings reported in Wedekind *et al.* (2013) suggest that such sex-specific mortality would have to be conditional to, or amplified at, certain temperature regimes.

Any form of environmental sex reversal would produce genotype-phenotype mismatches that should create strong differences in family sex ratios. If masculinisation or feminization led to biased population sex ratios, XX males would be expected to produce only daughters, or XY females, YY females, or YY males to produce only sons in laboratory experiments like the present one. However, no paternal or maternal effects on family sex ratio were found within a sample of 26 genitors (6 females and 20 males). This suggests that there is no environmental sex reversal in at least one of the study

populations, or that its prevalence is very low. The fact that no significant deviation from equal sex ratios could be found in the other study populations suggests that environmental sex reversal does not happen or is negligible.

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References

Afonso, L., Basu, N., Nakano, K., Devlin, R. & Iwama, G. (2003). Sex-related differences in the organismal and cellular stress response in juvenile salmon exposed to treated bleached kraft mill effluent. *Fish Physiology and Biochemistry* **29**, 173-179.

Azuma, T., Takeda, K., Doi, T., Muto, K., Akutsu, M., Sawada, M. & Adachi, S. (2004). The influence of temperature on sex determination in sockeye salmon *Oncorhynchus nerka*. *Aquaculture* **234**, 461-473.

Baroiller, J. F., d'Cotta, H. & Saillant, E. (2009). Environmental effects on fish sex determination and differentiation. *Sexual Development* **3**, 118-135.

Conover, D. O. (1984). Adaptive significance of temperature-dependent sex determination in a fish. *American Naturalist* **123**, 297-313.

Consuegra, S. & de Leaniz, C. G. (2007). Fluctuating sex ratios, but no sex-biased dispersal, in a promiscuous fish. *Evolutionary Ecology* **21**, 229-245.

Cotton, S. & Wedekind, C. (2009). Population consequences of environmental sex reversal. *Conservation Biology* **23**, 196-206.

Craig, J., Foote, C. & Wood, C. (1996). Evidence for temperature-dependent sex determination in sockeye salmon (*Oncorhynchus nerka*). *Canadian Journal of Fisheries and Aquatic Sciences* **53**, 141-147.

Davidson, W. S., Huang, T., Fujiki, K., von Schalburg, K. R. & Koop, B. F. (2009). The sex determining loci and sex chromosomes in the family Salmonidae. *Sexual Development* **3**, 78-87.

Devlin, R. & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* **208**, 191-364.

Esteve, M. & McLennan, D. A. (2007). The phylogeny of *Oncorhynchus* (Euteleostei : Salmonidae) based on behavioral and life history characters. *Copeia* **3**, 520-533.

Guerrero, R. & Shelton, W. L. (1974). An aceto carmine squash method for sexing juvenile fish. *The Progressive Fish-Culturist* **36**, 56.

Gum, B., Gross, M., Rottmann, O., Schroder, W. & Kuhn, R. (2003). Microsatellite variation in Bavarian populations of European grayling (*Thymallus thymallus*): Implications for conservation. *Conservation Genetics* **4**, 659-672.

Hari, R., Livingstone, D., Siber, R., Burkhardt Holm, P. & Güttinger, H. (2006). Consequences of climatic change for water temperature and brown trout populations in alpine rivers and streams. *Global Change Biology* **12**, 10-26.

Haugen, T. & Vøllestad, L. (2000). Population differences in early life-history traits in grayling. *Journal of Evolutionary Biology* **13**, 897-905.

243 Hunter, G., Solar, I., Baker, I. & Donaldson, E. (1986). Feminization of coho salmon
244 (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha*) by
245 immersion of alevins in a solution ofestradiol-17β. *Aquaculture* **3-4**, 295-302.
246 Jacob, A., Evanno, G., von Siebenthal, B. A., Grossen, C. & C. Wedekind, C. (2010). Effects
247 of different mating scenarios on embryo viability in brown trout. *Molecular Ecology*
248 **19**, 5296-5307.
249 Koop, B. F., von Schalburg, K. R., Leong, J., Walker, N., Lieph, R., Cooper, G. A., Robb, A.,
250 Beetz-Sargent, M., Holt, R. A., Moore, R., Brahmbhatt, S., Rosner, J., Rexroad, C. E.,
251 McGowan, C. R. & Davidson, W. S. (2008). A salmonid EST genomic study: genes,
252 duplications, phylogeny and microarrays. *BMC Genomics* **9**, 545. doi:10.1186/1471-
253 2164-9-545
254 Koskinen, M. T. & Primmer, C. R. (2001). High throughput analysis of 17 microsatellite
255 loci in grayling (*Thymallus* spp. Salmonidae). *Conservation Genetics* **2**, 173-177.
256 Magerhans, A., Mueller-Belecke, A. & Hoerstgen-Schwark, G. (2009). Effect of rearing
257 temperatures post hatching on sex ratios of rainbow trout (*Oncorhynchus mykiss*)
258 populations. *Aquaculture* **294**, 25-29.
259 Marshall, T. C., Slate, J., Kruuk, L. E. B. & Pemberton, J. M. (1998). Statistical confidence
260 for likelihood-based paternity inference in natural populations. *Molecular Ecology* **7**,
261 639-655.
262 Ospina-Álvarez, N. & Piferrer, F. (2008). Temperature-dependent sex determination in
263 fish revisited: Prevalence, a single sex ratio response pattern, and possible effects of
264 climate change. *PLoS One* **3**, e2837.
265 Pickering, A. & Willoughby, L. (1982). *Saprolegna* infections of salmonid fish. In
266 *Microbial diseases of fish* (Roberts, R., ed.), pp. 271-297. New York, USA: Academic
267 Press.
268 Pompini, M., Clark, E. S. & Wedekind, C. (2013). Pathogen-induced hatching and
269 population-specific life-history response to water-borne cues in brown trout (*Salmo*
270 *trutta*). *Behavioral Ecology and Sociobiology* **67**, 649-656.
271 Poulin, R. & Thomas, F. (1999). Phenotypic variability induced by parasites: Extent and
272 evolutionary implications. *Parasitology Today* **15**, 28-32.
273 R Development Core Team (2011). R: a language and environment for statistical
274 computing. Vienna, Austria: R foundation for statistical computing.
275 Schartl, M. (2004). Sex chromosome evolution in non-mammalian vertebrates. *Current*
276 *Opinion in Genetics & Development* **14**, 634-641.
277 Stelkens, R. & Wedekind, C. (2010). Environmental sex reversal, trojan sex genes, and
278 sex ratio adjustment: conditions and population consequences. *Molecular Ecology* **19**,
279 627-646.
280 van den Hurk, R. & Slof, G. A. (1981). A morphological and experimental study of gonadal
281 sex differentiation in the rainbow trout, *Salmo gairdneri*. *Cell and Tissue Research* **218**,
282 487-497.
283 von Siebenthal, B. A., Jacob, A. & Wedekind, C. (2009). Tolerance of whitefish embryos to
284 Pseudomonas fluorescens linked to genetic and maternal effects, and reduced by
285 previous exposure. *Fish and Shellfish Immunology* **26**, 531-535.
286 Wedekind, C. (2012). Managing population sex ratios in conservation practice: how and
287 why? In *Topics in Conservation Biology* (Povilitis, T., ed.), pp. 81-96. InTech Open
288 Access Publisher. doi: 10.5772/37601
289 Wedekind, C., Evanno, G., Székely, T., Pompini, M., Darbellay, O. & Guthruf, J. (2013).
290 Persistent unequal sex ratio in a population of grayling (Salmonidae) and possible
291 role of temperature increase. *Conservation Biology* **27**, 229-234.

292 Wedekind, C. & Küng, C. (2010). Shift of spawning season and effects of climate warming
293 on developmental stages of a grayling (Salmonidae). *Conservation Biology* **24**, 1418-
294 1423.
295 Yano, A., Nicol, B., Jouanno, E., Quillet, E., Fostier, A., Guyomard, R. & Guiguen Y. (2013)
296 The sexually dimorphic on the Y-chromosome gene (*sdY*) is a conserved male-specific
297 Y-chromosome sequence in many salmonids. *Evolutionary Applications* **6**, 486-496.
298

299 **TABLE I.** Maternal, paternal, and treatment (warm vs. cold water) effects on offspring sex
300 of *T. thymallus* raised in the 2010 experiment (analysis of deviance table for generalized
301 linear model; N_{total} = 495).
302

Factor	d.f.	Deviance	*P*
Dam	5	9.2	0.10
Sire	19	19.1	0.45
Treatment	1	0.0	0.89
Treatment x Dam	5	2.2	0.83
Treatment x Sire	19	16.3	0.64

303

Supporting Information: "Temperature-induced sex reversal is not responsible for sex ratio distortions in grayling *Thymallus thymallus* or brown trout *Salmo trutta.* – M. Pompini, A. M. Buser, M. R. Thali, B. A. von Siebenthal, S. Nusslé, S. Guduff, and C. Wedekind**"**

SI. Supplementary table I

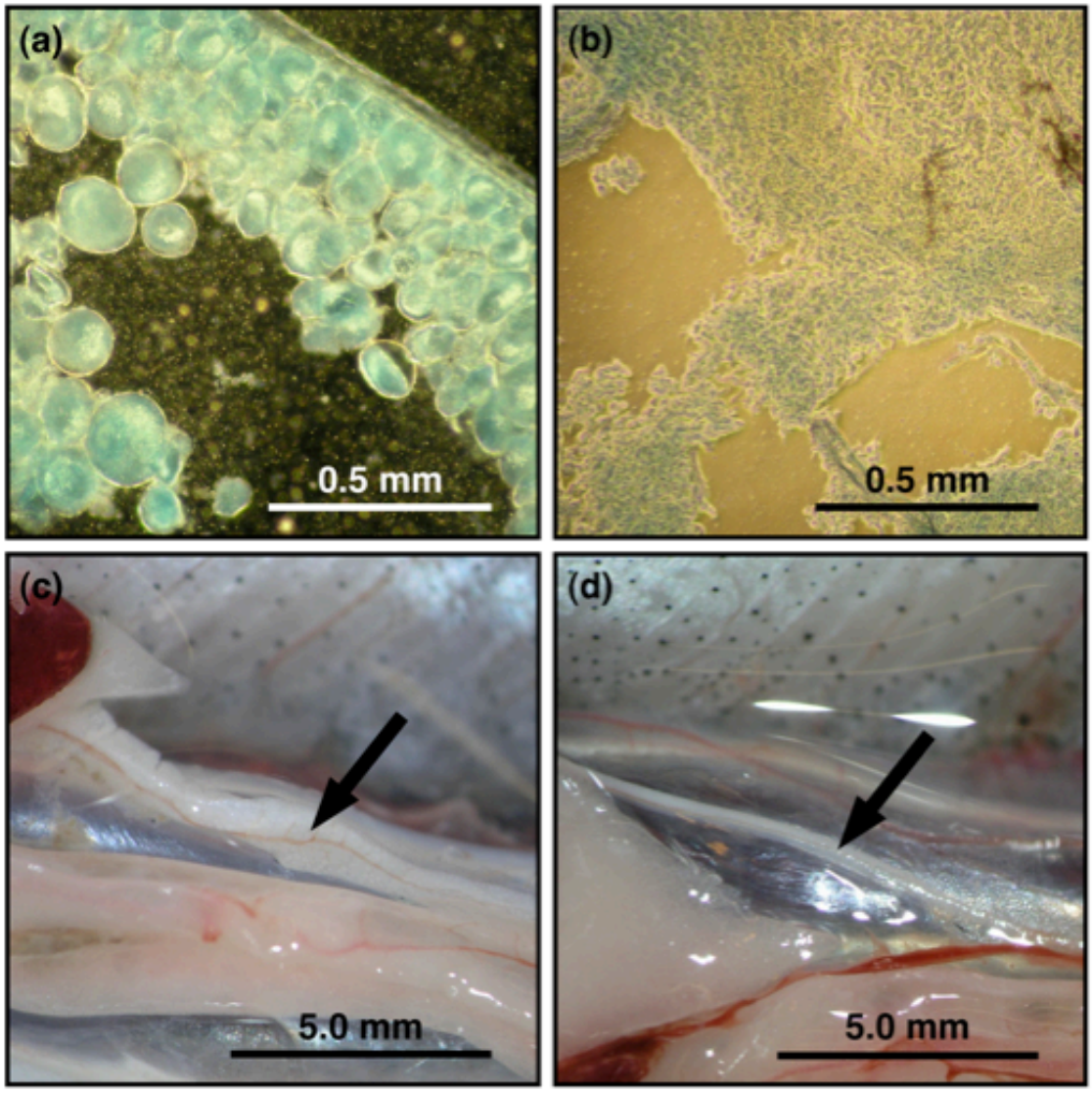
SUPPLEMENTARY TABLE I. Origin of samples, number of genitors, experimental breeding, temperature treatment, number of sexed offspring (of the total number of F1 individuals that could be successfully raised), mortality from fertilization until the time of sexing, and observed sex ratio (% males) of experiments with (a) *T. thymallus*, and (b) *S. trutta*. ATU = accumulated thermal units, i.e. degreedays.

Population (year), Genitors	Experimental design	Temperature treatment	N sexed (total)	Mortality	Sex ratio
a) T. thymallus					
Aare (2007) 7♀, 36♂	Full-factorial *in vitro* fertilizations (females x males): 3 x 30 and 4 x 6; offspring split about equally to 4 different temperature treatments and raised until phenotypic sex could be determined	Constant 6°C until 60 ATU, constant 5°C from 60-300 ATU, 8-15°C from then on	609 (~4,500)	<5%	48.0%
		Constant 6°C until 60 ATU, constant 7°C from 60-300 ATU, 8-15°C from then on	107 (~4,500)	<5%	44.9%
		Constant 6°C until 60 ATU, constant 9°C from 60-300 ATU, 8-15°C from then on	112 (~4,500)	<5%	49.1%
		Constant 6°C until 60 ATU, constant 11°C from 60-300 ATU, 8°-15°C from then on	318 (~4,500)	<5%	47.5%
Rhine (2008) 3♀, 24♂	Full-factorial *in vitro* fertilizations: 3 x 24; offspring split about equally to 2 different temperature treatments and raised until phenotypic sex could be determined (starting with in total 2,880 embryos raised in 120 24-well plates)	Constant 6°C until 120 ATU, continuously increasing from 6°C - 8°C between 180-300 ATU, with a 1°C daily variation simulating day and night, 14°C from then on	254 (254)	82.4%	47.2%
		Constant 6°C until 120 ATU, continuously increasing from 6°C - 12°C between 180-300 ATU, with a 1°C daily variation simulating day and night, 14°C from then on	0 (0)	100%	.

Rhine (2009) 6♀, 20♂	Full-factorial *in vitro* fertilizations: 6 x 20; offspring split about equally to 2 different temperature treatments, from 1000 ATU on raised at two different locations (starting with in total 4,800 embryos raised in 200 24-well plates)	Constant 5°C until 1,000 ATU, constant 14°C from then on	27 (27)	97.8%	63.0%
		Constant 10°C until 1,000 ATU, constant 14°C from then on	187 (187)	84.4%	46.0%
		Constant 5°C until 1,000 ATU, ca 15°C from then on	0 (0)	100%	.
		Constant 10°C until 1 000 ATU, ca 15°C from then on	119 (119)	90.1%	52.1%
Rhine (2010) 6♀, 20♂	Full-factorial *in vitro* fertilizations: 6 x 20; offspring split about equally to 2 different temperature treatments (starting with in total 4,800 embryos raised in 200 24-well plates)	Continuously increasing from 6°C - 14.5°C until 1,000 ATU, 8°- 9°C from then on	245 (245)	89.8%	47.0%
		Continuously increasing from 8.5°C -15°C until 1,000 ATU, 8°- 9°C from then on	250 (~1,500)	39.6%	48.8%
Aare (2011, early season) 2♀, 10♂	Full-factorial *in vitro* fertilizations: 2 x 10 (starting with in total 2,400 embryos raised in 100 24-well plates)	Constant 5°C until 1,000 ATU, 8° - 9°C from then on	34 (34)	98.3%	55.8%
Aare (2011, late season) 2♀, 10♂	Full-factorial *in vitro* fertilizations: 2 x 10 (starting with in total 2,400 embryos raised in 100 24-well plates)	Constant 10°C until 1,000 ATU, 8° - 9°C from then on	100 (~1,600)	20%	46.0%
b) S. trutta					
Aare and 4 tributaries (2009)	Full-factorial *in vitro* fertilizations within populations, i.e. 5	Continuously declining from 6.5°C to 1°C until 400 ATU, then continuously	339 (339)	85.6%	52.8%

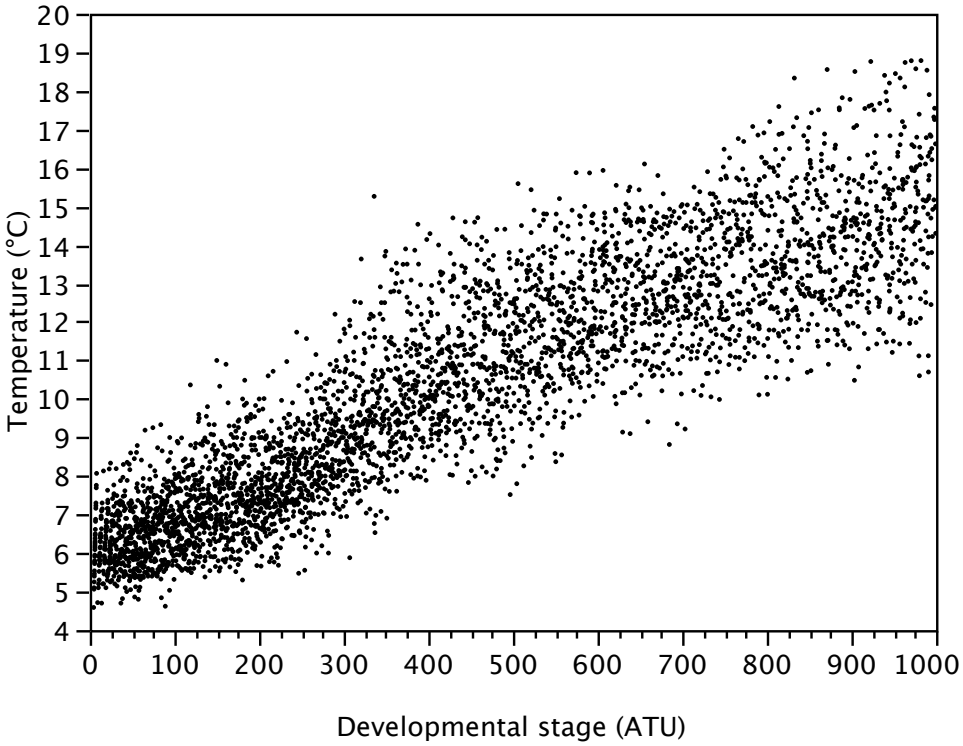
20♀, 30♂	times 4 females x 6 males; offspring about equally split to 2 temperature treatments (starting with in total 4,800 embryos raised in 200 24-well plates)	increasing to 7°C until 1,000 ATU, then 8°-9°C			
		Continuously increasing from 6.5°C to 14.5°C until 1 000 ATU, 8°C to 9°C from then on	208 (208)	91.8%	47.6%

SII. Supplementary Figure 1



Supplementary Figure 1. Examples of gonads of *T. thymallus* after 6 months, with (a) oocytes, (b) spermatocytes, and phenotypes of (c) female and (d) male gonads.

SIII. Supplementary Figure 2



Supplementary Figure 2. The range of water temperature (in °C) that embryos and early larvae experienced at the natural spawning site in the River Aare when gamete fusion was at the peak of the spawning season, i.e. when half of all females of the respective season had spawned. Each dot represents a daily average temperature during the first 1 000 ATU of embryo and larval development, as measured from spring 1971 until spring 2011. Mean hatching from egg in the 2009 *T. thymallus* experiment was around 206.6 ATU (SD = 7.3).